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(54) Food products containing bacteria with cholesterol lowering activity

(57) The invention concerns bacteria having byle salt hydrolysis activity and optionally and preferably also bile salt polymerisation activity. Food products comprising such bacteria have been found to be capable to reduce the blood cholesterol level in the human blood. The effect is obtained by interference with the cholesterol and/or bile salt metabolism and is based on BSH activity of the bacteria. Food products which, on the basis of their normal daily intake provide a daily-BSH-intake of 0.3 micro-mol/min/kg bodyweight, are preferred, and even more preferred are food products providing at least a daily-BSH-intake of 0.6 micro-mol/min/kg bodyweight. In a further preferred embodiment a BSH activity of at least 0.8 micro-mol/min/1e10 cfu is obtained by the common, daily intake of food products comprising the bacteria. Further preferred are food products comprising fat, wherein at least 40% of the fat are poly unsaturated fatty acids.

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of bacteria is expressed as micro-mol of free bile salts formed per minute per 1e10 colony forming units (cfu) of viable bacterial cells (micro-mol/min/1e10cfu). Therefore, the preferred daily BSH intake is expressed as micro-mol/min/kg bodyweight. We have found that the minimum preferred daily-BSH-intake supplied by the probiotic food product is 0.3 micro-mol/min/kg of bodyweight. Assuming that at least 2%, preferably at least 20%, of the bacteria survive the passage through the stomach the preferred daily-BSH-intake provided by the probiotic food will be sufficient to show a significant reduction in blood cholesterol.

In prior art described wild type bacteria, selected in order to test the hypothesis described in Introduction, posses BSH-activities around 0.10 micro-mol/min/1e10 cfu. We have now found that these activities are not sufficient to reach the required daily-BSH-intake. For example, an average person of 70 kg would need to consume at least 2.1e12 cfu per day of these bacterial cells to reach the preferred daily-BSH-intake as described in this invention. It will not be possible to provide such amounts of bacteria in normal consumer food products for day to day use in a cost effective way. The bacteria we selected posses BSH-activities of at least 0.80 micro-mol/min/1e10cfu, preferably at least 1.50 micro-mol/min/1e10cfu, thereby reducing the amount of cells needed by a factor 15 (1.4e11cfu per day), which makes the application in consumer food products more feasible.

In another preferred embodiment of this invention it is claimed that intake of probiotic cells with BSH activity is accompanied by another activity, namely the bacterial activity to polymerise the deconjungated bile salts, which in turn are formed upon hydrolysis by BSH activity, for example polymerisation of deoxycholate to 3-alpha-poly-deoxycholate. Preferably these activities are combined in one bacterial strain, however, a probiotic food product consisting of one or more strains in that way providing sufficient levels of both activities will lower blood cholesterol as well.

The polymerised deconjungated bile salts formed by polymerisation activity of the bacterial cells will not be absorbed in the human small intestine, while deconjungated bile salts, formed by BSH-activity, are less readily absorbed than their tauro- or glyco-conjungated equivalents. That means that a combination of polymerisation activity and BSH activity will very effectively interfere with the cholesterol and/or bile salt metabolism in the human body. Therefore when these activities are supplied in combination in a probiotic food product, less daily-BSH-intake as such will be required The lower daily-BSH-intake will then be compensated by the presence of polymerisation activity which leads to the complete inhibition of the reabsorption of the formed deconjungated bile salts. In this invention, the polymerisation activity of bacterial cells is expressed as micromol of deconjungated bile salts polymerised per min per 1e10 cfu (micro-mol/min/1e10cfu). The claimed probiotic food product will therefore supply the sum of both activities at a minimum level of 0.3 micro-mol/min/kg bodyweight, while the ratio of BSH activity and polymerisation activity should preferably by greater than unity. Preferably, a minimum level of 0.6 micro-mol/min/kg bodyweight is supplied.

In another preferred embodiment of this invention, BSH-activity alone or together with polymerisation activity in probiotic food products is combined with other cholesterol lowering substances like polyunsaturated fatty acids as currently present in heart health fat based food products, providing that the food product supplies a minimum level of both activities as defined above.
In particular, fat containing food products of which the fat comprises at least 40% polyunsaturated fatty acids have been found to be very beneficial in this respect.

EXAMPLES

Example I

HPLC determination of BSH-activity

Various bacteria were grown as a stirred culture on MRS (Difco) supplemented with $CaCO_3$ (1g/l) and anaerobic conditions (CO_2 on headspace). The final cultures were centrifuged (10000*g*) and concentrated to approximately 80 folds of the original broth volume. The cell pellet was mixed in equal amounts (w/w) with a cold solution of non fat dry milk (20% (w/w)) and stored at -30°C. After storage for one night the experiments were carried out with a fresh prepared solution of the frozen samples diluted in 10 mM sodium-acetate buffer (pH 5.6) to OD610 = 20 . To 0.5 ml of this solution, 0.5 ml 32 mM TCA-solution was added. The reaction tubes were placed in a waterbath at 37°C. After incubation for 5, 10, 15 or 30 minutes, the reaction was stopped by pasteurization of the reaction mixture (5 min, 90°C). After removing the biomass by centrifugation, the supernatant was filtered (0.8 µm) and stored at -30°C. The time-zero samples were prepared by pasteurizing the solution and additionally adding the substrate solution. Viable counts of the solution were performed on MRS agar plates after 10-fold dilution in peptone-physiological saline (1.0 g/l peptone, 8.5 g/l NaCl).

Taurocholic acid and cholic acid in the incubation mixture were separated by reversed-phase HPLC on a PLRP-S 100Å, 5µ, 150*4.6 mm column (Polymer Laboratories). The eluens was composed of 22 % acetonitrile in 0.1 M NaOH and it was used at a flow rate of 1.5 ml/min. The analytes were detected with a Decade pulsed amperometric detector (Antec-Leyden)equipped with a gold working electrode and an Ag/AgCl reference electrode. The pulse programme of the detector included three potentials: E1=0.03V with a duration time of 1.6 s (measuring potential), E2=0.6V with a duration time of 0.3 s (cleaning potential) and E3=-0.8V with a duration time of 0.3 sec (reduction of gold electrode surface). The column temperature was maintained at 35°C by a column thermostat. Samples were filtered through

dried for 1 hour at 110°C. 10 µl of sample was applied and plates were developed in methanol-acetonitrile-water-formic acid 45:45:10:0.5 (v/v). After development, the eluent was evaporated and the plates were dried for 10 minutes at 110°C. To visualize free and polymerised bile acids, the plates were sprayed evenly with a mixture of methanol-water-sulphuric acid-$MnCl_2.4H_2O$ (150 ml/150 ml/10 ml/1 g) and dried at 110°C for 15 minutes. Free and polymerised bile salts were visualized as fluorescent bands in UV light (254 nm). Polymerised bile salts remained at the bottom of the TLC plate.

Example IV

Animal trial with rat as model system

Twelve male rats (250g on average) were fed a semi-synthetic diet containing 0.1% cholesterol. Ten of the rats were ileostomized. After a recovery period of 14 days, fasted blood samples, ileal digesta samples and a 48 hr collection of excreted faeces were taken. Probiotic bacteria were then added to the diet daily. After 14 days of probiotic feeding, ileal digesta, a blood sample and a 48hr faecal collection were taken. For a further seven days rats were fed without addition of probiotic and ileal digesta, a blood sample and a 48hr faecal collection were again taken. The diet was then changed to contain 1.0% cholesterol and the sampling procedures for ileal digesta, blood and faecal collections were repeated before, during and after a 14 day period of probiotic feeding. The diets were mixed with deionized water (in the ratio; 1.5/1, water/diet) just before feeding.

The bacteria used in this experiment is a *Lactobacillus animalis* (strain 364) isolated from hamster faeces. Strain 364 was identified as a *L.animalis* strain by SDS-page analysis. Strain 364 has a very high BSH activity (0.8 micro-mol/min/1e10cfu), compared with other intestinal strains, and was additionally subselected to possess resistance to streptomycin. The bacteria were grown as a stirred 10L and free acidifying culture for approximately 16hr at 34°C using MRS-medium supplemented with $CaCO_3$ (1g/l). For preparation of the feeding samples, the collected cell pellet (80 times concentrated) was mixed in equal amounts (w/w) with a cold solution consisting of 20% (w/w) non fat dry milk and stored at -30°C.

The bacteria were given as an addition to the food of the rats. Because the bacteria are prepared in an aqueous suspension it was necessary to feed the diet mixed with water. The bacteria were supplied frozen in vials sufficient for feeding all the rats at each meal. The viability and activity of the bacteria after thawing and the stability of the bacteria in their BSH-activity in the diet was controlled and found stable.

The amount of food required per meal for the group of rats was calculated from the food consumption data obtained in the first 7 days of the study, plus 10% for spillage and wastage. The amount of bacteria per vial, to be mixed with food and water, was calculated to provide 10^{11} live bacteria per day.

Blood samples were taken before the morning feeding from the cut tip of the tail. Serum was prepared and total cholesterol measured. The ileal samples were taken from anaesthetized rats at approximately 12.00 hr before, during and after probiotic feeding.

There was no difference in growth rate and food intake between periods of control and probiotic feeding. There was no incidence of ill-health associated with probiotic feeding. The number of lactobacilli found in ileal digesta when grown on MRS agar, with and without streptomycin were analyses. There was a small increase in total lactobacilli during feeding of strain 364 on both low and high cholesterol diets. There was however a much larger increase in streptomycin resistant lactobacilli during feeding of strain 364. It was calculated that more than 20% of the bacteria fed survived the passage through the stomach.

When fed the low cholesterol diet there was approximately a 6% reduction in blood cholesterol levels during bacteria feeding (from 2.45 to 2.30 mM) and when fed the high cholesterol diet there was approximately a 7% reduction (from 2.78 to 2.58 mM). This cholesterol reduction in rat was achieved with a daily-BSH-intake of 32 micro-mol/min/kg bodyweight. Polymerised bile salts were found in higher amounts in the faeces of the rats in the probiotic feeding group than in the control group, as visually and qualitatively determined with the procedure as described above.

Example V

Animal trial with pigs as model system

Twenty pigs (10 females and 10 castrated males) of about 30 kg live weight at the start of the experiment, age 10 weeks, were divided into two experimental groups, the control pigs and the pigs to be treated with the probiotic supplement. The allocation of the pigs to one of the groups was performed in such a way that both groups showed equal distributions of the sexes, equal initial weights and cholesterol levels. The pigs were inspected daily for any evidence of diarrhoea, constipation or other illnesses or observations. The pigs were weighed at the beginning and end of each experimental period.

The experiment lasted for 13 weeks. During the first five weeks, all pigs received a diet rich in saturated fat and low in fibre content, which contained 0.2% (w/w) cholesterol. During the following five weeks, the animals were fed the same diet in which the cholesterol content was doubled (0.4% w/w). This high-fat diet was supposed to initially lead to increased serum cholesterol levels. During probiotic feeding, from week 4 up to and including week 7, the treated group received the probiotic strain both in the morning and evening feed. During the last three weeks, all animals received a regular diet without cholesterol addition.

The *Lactobacillus* strain used was isolated from

B.infantis was characterised as 1.79 micro-mol/min/1e10 cfu.

To obtain a spread, a similar procedure as described in previous example was followed. However, to 27 parts of water, 2 parts of the *B.infantis* concentrate was added. Other processing was similair than in previous example. A good fat continuous spread was obtained. It contains 57% PUFA on fat blend and 8e9 cfu/g product of *B.infantis*. The viable count remained stable during storage at 5°C for at least 10 weeks.

Example VIII

Preparation of a dressing

15 parts of pasteurized drink yoghurt is mixed with 2 parts of acidic acid (10%), 10 parts of sugar, 5 parts of *B.infantis* concentrate as described above, and 43 parts of water. To this mixture 10 parts of various flavour components, preservatives, thickeners and emulsifiers are added. The mixture is thouroughly mixed in a stainless steel stirred vessel. To this aquous mixture 15 parts of sunflower oil is added, thourouhly mixed for an additional 15 min, to obtain a pre-emulsion. The pre-emulsion is brought into a colloid mill (Prestomill PM30) and processed at a split-size between level 15 and 20 and a throughput between level 4 and 6. A good water continuous dressing was obtained. It contains 2e10 cfu/g product of *B.infantis*. The viable count remained stable during storage at 5°C for at least 7 weeks.

Claims

1. Food products which lower blood cholesterol based on the presence of bacteria which after gastric transit will interfere with the cholesterol and/or bile salt metabolism and were the interference with cholesterol and/or bile salt metabolism is based on bile salt hydrolysis activity of the bacteria.

2. Food products according to claim 1 which provide at least a daily-bile salt hydrolysis-intake of 0.3 micro-mol/min/kg bodyweight.

3. Food products according to claim 2 which provide at least a daily-bile salt hydrolysis-intake of 0.6 micro-mol/min/kg bodyweight.

4. Food products according to claim 3, which contain bacteria with a bile salt hydrolysis activity of at least 0.8 micro-mol/min/1e10 cfu.

5. Food products according to claim 1, where the interference with cholesterol and/or bile salt metabolism is additionally based on free bile salt polymerisation activity of foodgrade bacteria.

6. Food products according to claim 5, which provide at least a daily combined bile salt hydrolysis and free bile salt polymerisation activity of 0.3 micro-mol/min/kg bodyweight.

7. Bacteria selected on their bile salt hydrolysis activity, with bile salt hydrolysis activities sufficient enough to be used in foods in order to interfere with the cholesterol and/or bile salt metabolism in the human body.

8. Bacteria according to claim 7 with a bile salt hydrolysis activity of at least 0.8 micro-mol/min/1e10cfu.

9. Bacteria according to claim 7 with a combined bile salt hydrolysis and bile-salt-polymerisation activity.

10. Fat containing food products according to any one of claims 1 to 6, wherein the fat in the product comprises at least 40% of poly-unsaturated fatty acids.